



ESTIMATION AND CHARACTERIZATION OF CYCLOPROPENOID FATTY ACIDS

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

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IN

CHEMISTRY

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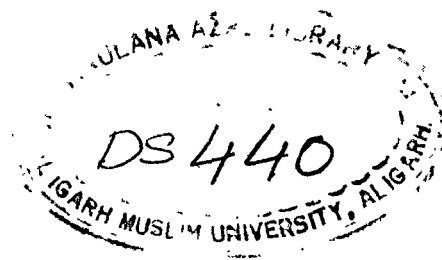
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Rafi Ahmad
(RAFI AHMAD)

**Dedicated
to
My Parents**

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INTRODUCTION

Fatty acids are an essential structural component of nearly all compounds embraced by the term lipid. From early 1950's the most significant advances in the field of lipid research have been the discovery of naturally occurring cyclopropenoid fatty acids (CPFA) in higher plants. The CPFA have recently been the subject of intensive investigation and have been shown to produce numerous physiological disorders in farm and laboratory animals. Because of their biological effects, the quantitation of CPFA in seed glycerides is of great importance.

The CPFA autoxidize and polymerize very readily in air at room temperature. So the techniques of isolation and analysis must be such as to minimize ill effects from such cases.

Several methods have been developed for the separation and quantitation of CPFA but the absolute quantity of these unusual acids in seed lipids has not so far been demonstrated by any reliable method.

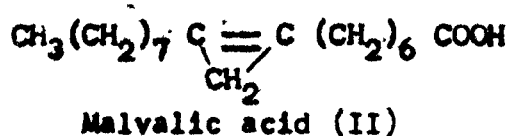
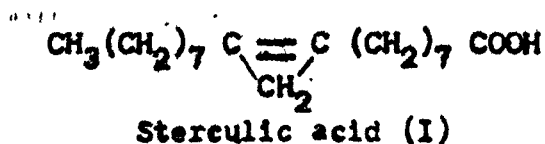
When the presence of CPFA in the seed lipid is suspected, the intact lipids should be examined as fully as possible by non-destructive techniques. The spectroscopic

techniques constitute a back bone of modern chemical investigations. They are used as routine analytical procedures for quality control in industries, for monitoring reactions, for probing structure of molecules, and for guidance in research and development. Electronic instrumentation viz., ultraviolet (uv), infrared (IR), nuclear magnetic resonance (NMR), mass spectrometry (MS) and gas liquid chromatography (GLC) are widely used. These non-destructive methods are less time consuming and find wide application for estimation and characterization of CPFA.

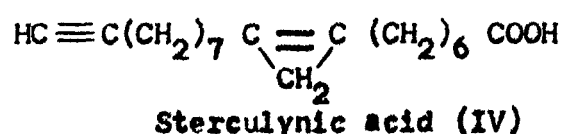
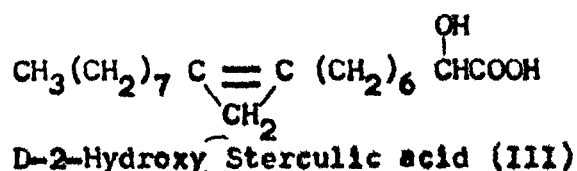
Keeping in view the special significance of CPFA, the present dissertation is an attempt to compile the literature on estimation and characterization of CPFA.

CURRENCI OF CYCLOPROPENOID FATTY ACIDS

The 1,2-disubstituted cyclopropene function occurs in the fatty acid chain of lipids from certain plants belonging to the order Malvales (Malvaceae, Sterculiaceae, Tiliaceae and Bombacaceae families),^{1,2} cotton seed oil being the most common. In 1952, Nunn first isolated the cyclopropenoid fatty acid (CPFA) from Sterculia foetida (Sterculiaceae)³ oil and named sterculic acid (9,10-methyleneoctadec-9-enoic acid) (I) one of these fatty acids, malvalic acid (8,9-methyleneheptadec-8-enoic acid) (II) is a component of cotton seed oil triglycerides⁴. Malvalic acid was first isolated and characterised by MacFarlane et al.⁵ and recognised as a homologue of sterculic acid. Both the acids, viz., sterculic and malvalic often occur together and sometimes may be accompanied by small amount of their dihydro derivatives. In general, the quantity of sterculic acid predominates in sterculiaceae and that of malvalic acid predominates in Malvaceae seed oils.



Two other cyclopropenoid acids have been discovered; D-2-hydroxy-sterculic acid (III) in the seed oil of Paqura insignis (Bombacaceae)⁶ and Sterculynic (8,9-methylene octadec-8-ene-17-ynoic) acid (IV) in the seed oil of Sterculia alata (Sterculiaceae)⁷.



Over a period of years, others investigators have found in various species indications of elusive cyclopropenes with chain lengths shorter than malvalic acid. Raju and Reiser⁸ reported evidence for a CPFA with a GLC retention time shorter than malvalic acid in Althaea rosea (Malvaceae) seed oil. Johnson *et al.*⁹ hinted the occurrence of C₁₇ cyclopropenes in the fruits of certain Malva species. Ackman and Hooper¹⁰ encountered an unusual component in Euphorbia longana (Sapindaceae) seed oil which they regarded as possibly being a C₁₁ CPFA with a cyclopropene function near the methyl end. Among the recent and comprehensive additions to the literature of CPFA has been those of Madrigal *et al.*¹¹ and according to them Pavonia spium seed oil is unusual in being the first of the family

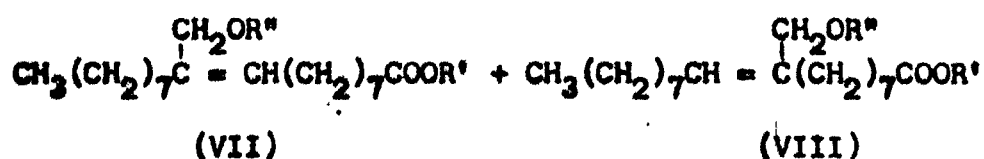
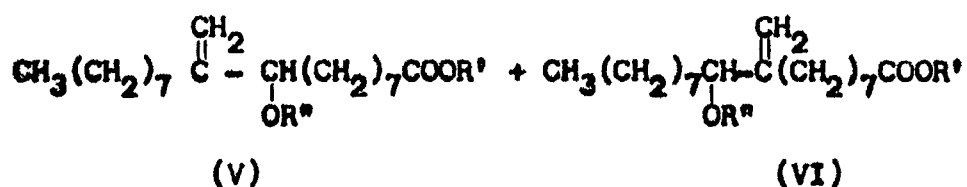
Malvaceae in which the content of sterculic acid was observed to be greater than that of malvalic acid disobeying the general trend of the family. In addition the seed oil of Pterospermum acerifolium (Sterculiaceae)¹² is also unusual in containing the malvalic acid in greater quantity than that of Sterculic acid. More recently, Ahmad et al.¹³ reported Eriolaena hookeriana, the second unusual seed oil of the family Sterculiaceae in which they observed the content of malvalic acid (25.8%) to be greater than sterculic acid (6.0%),

Dihydrosterculic (cis-9,10-methylene-octadecanoic) acid was found in the seed oil of Euphorbia longana (Sapindaceae)¹⁴. Lie Ken Jie et al.¹⁵ discovered the co-occurrence of dihydrosterculic and cis-9,10-methylene-hexadecanoic acids in Lychee (Litchi sinensis, F. Sapindaceae) seed oil. An investigation of the seed oil of Byrsonia coccinea (Connaraceae)¹⁶ disclosed the presence of lactobacillic (cis-11,12-methylene-octadecanoic) acid in its oil. Lactobacillic acid has long been known as a constituent of certain bacterial lipids, but this is the first report of its presence in a seed oil.

Because of their biological effects^{17,18} the quantitation of CPFA is of great importance. Several methods have been developed for the isolation and quantitation, but the absolute quantities of cyclopropenoid fatty acids in fats and oils have not so far been determined by any reliable method.

ISOLATION OF CYCLOPROPENOID FATTY ACIDS

The cyclopropenoid fatty acids, when kept in air at room temperature, autoxidise and polymerise very readily as indicated by the increase of the equivalent weight with time, and even slowly at 0°C. Faure *et al.*¹⁹ on the basis of IR analysis, suggested that sterculic acid polymerises with opening of the cyclopropene ring. Polymerisation proceeds via isomerisation of the cyclopropene ring with carboxylic acid addition to form a mixture of polyesters (V-VIII), as shown below.



R' = R'' = Sterculic acid residues.

The polymer gave no colour with the Halphen reagent. The IR spectrum of the purified polymer gave no absorption at 1852 and 1010 cm⁻¹ (cyclopropene ring). A new absorption band at 1737 and 1169 cm⁻¹ suggested the presence of ester, while bands at 1648 and 901 cm⁻¹, and at 1712 and 960 cm⁻¹ were indi-

native of unsymmetrical disubstituted olefin and terminal carboxyl groups, respectively. Therefore, the isolation technique and analysis must be such as to minimise ill-effects from such causes.

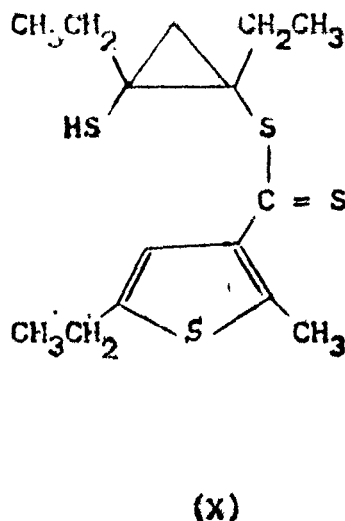
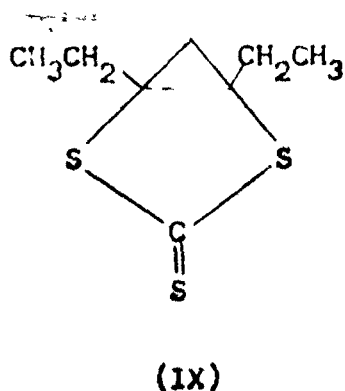
Literature survey did not mention any method which can be used for quantitative isolation of different cyclopropene fatty acids in a mixture. It is possible, however, by selecting suitable seed oils as starting materials to obtain individual CPFA of high purity. Nunn³ isolated sterculic acid of sufficient purity for structure determination by a combination of urea fractionation and low temperature fractional crystallisation of the fatty acids of S. foetida seed oil. In 1965, Fogerty et al.²⁰ used liquid liquid partition column chromatography to obtain pure methyl malvalate from a concentrate of the ester prepared by the urea fractionation technique from S. foetida esters or from Gossypium hirsutum (cotton seed) esters, although this contains only 1-2% of cyclopropenoid components. Jevans and Hopkins⁷ used counter current distribution technique to obtain pure sterculynic acid, but this technique does not appear to have been tried for other cyclopropenoid acids. In general, the isolation procedures used by the various group of workers in their studies followed either the urea complex fractionation or the low temperature fractional crystallisation methods.

DETECTION AND CHARACTERISATION OF CYCLOPROPENOID FATTY ACIDS

I) Halphen Test

This test was originally developed as an empirical method of testing the adulteration of various vegetable oils by cotton seed oil. A very small amount of a fatty acid with a cyclopropene ring was suggested as the material responsible to give red or orange colour. This reaction is now believed to be specific for cyclopropene ring and is quick and easy method of checking whether CPFA are present in a mixture or not.

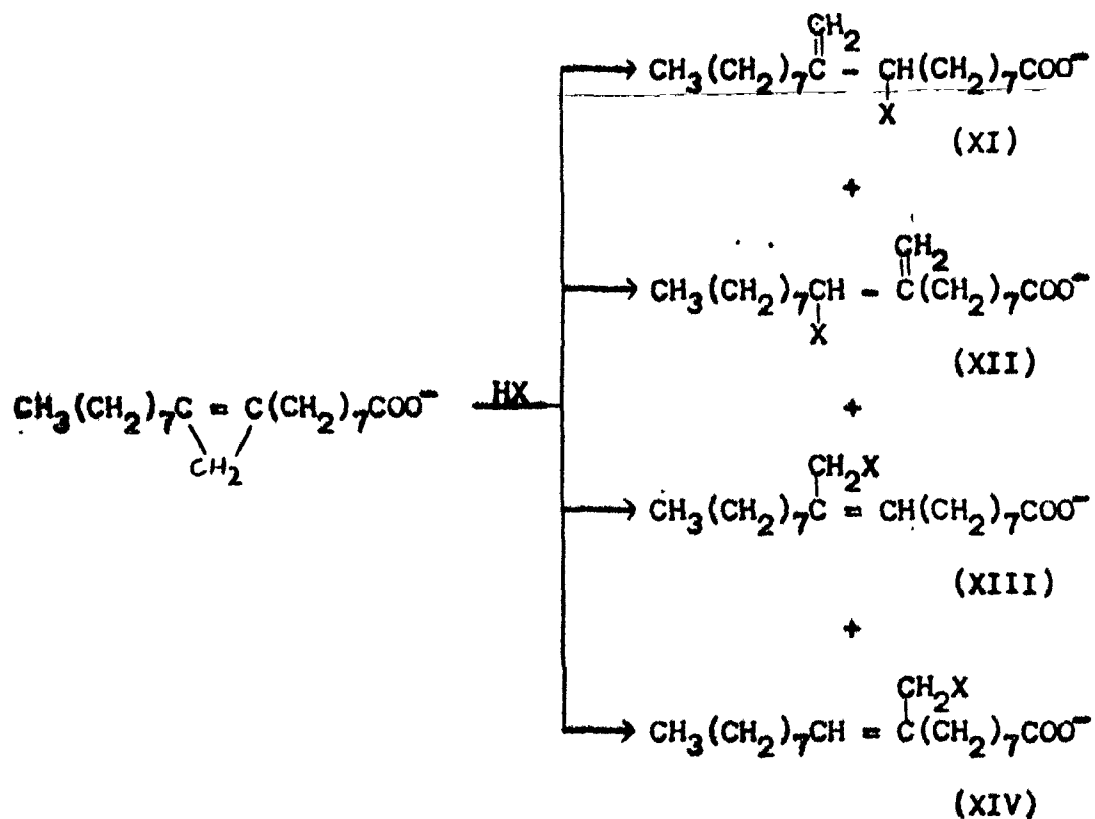
The Halphen reaction²¹ involves an opening of the ring across the single bonds. As the reaction proceeds and the colour develops, the characteristic infrared absorption bands of the cyclopropene ring (1852 and 1010 cm^{-1}) disappear and new ones appear at 1626 , 2050 and 772 cm^{-1} . Faure²² reported that the rapid appearance of a strong band at 2050 cm^{-1} and its subsequent disappearance are attributed to the double bond in the grouping -S-C=S , which was first formed by reaction between carbon disulphide and the cyclopropene ring, and afterwards polymerised across the C=S bonds. Zahorsky *et al.*²³ established the structures of two coloured product (IX,X) formed by reaction of 1,2-diethylcyclopropene with the Halphen reagent (1% solution of sulphur in carbon disulphide).



II) HBr - Titration

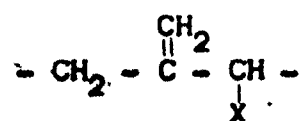
Cyclopropenoid fatty material reacts slowly but quantitatively with one mole of hydrogen bromide (HBr) in glacial acetic acid. This property is developed into a routine analytical technique for determining the total cyclopropenoid content of seed oils. The quantitative addition of a molecule of hydrogen chloride (HCl) to the cyclopropene ring, when the sample is shaken with concentrated hydrogen chloride, is the basis of another analytical method. The cyclopropenoid content was calculated from the increase in the chlorine content of the sample but this method is tedious and appears to be inferior to procedures using hydrogen bromide.

The reaction of HCl or HBr with the cyclopropene ring involves the formation of four isomeric monounsaturated monohalomoieties (XI-XIV) as shown below :



X = Br or Cl.

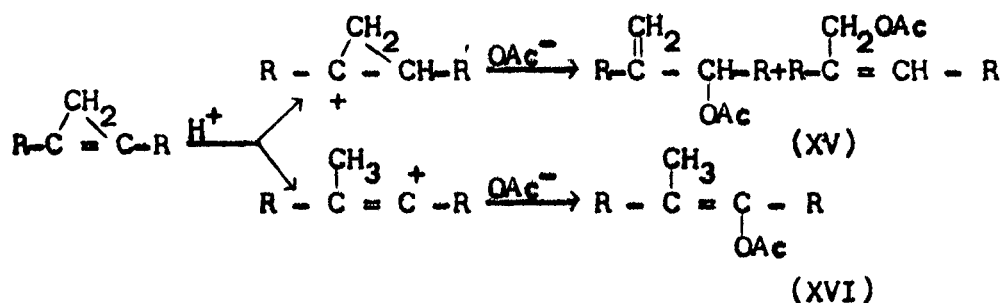
The structures of the reaction products have been investigated by Bailey *et al.*²⁴ using infrared spectroscopy. The reaction products gave a strong IR band at 905 cm^{-1} attributable to the group



and the mechanism of the reaction appears to involve opening

of the ring at one of the single bonds with concomitant addition of HX.

A more recent study of the reaction of sterculene with acetic acid, carried out by Kircher²⁵, showed that two mechanisms operate leading to different type of products, viz., protonation of one of the olefinic carbon atoms of the cyclopropene ring followed by ring opening and addition of the acetate ion to form allylic esters (XV); or protonation of the methylene group, again with ring opening, and addition of acetate ion to form the enol ester (XVI), as shown below :



Thus, it has been realised that the determination of total cyclopropenoid content by titration with HBr in acetic acid solvent at 55°C is liable to give incorrect results. This has been shown to be caused by the reaction of upto 17% of the cyclopropenoid material present with the acetic acid solvent during 20 minute - titration time. To overcome this, Rosie *et al.*²⁶ demonstrated a modified method for the determination of total cyclopropenoid material by reaction with HBr in benzene medium. Contrary to previous methods, this reaction

proceeds rapidly at room temperature and obviate the undesirable need for the use of elevated temperatures in total cyclopropenoid determination.

III) Gas Liquid Chromatography (GLC)

The GLC of cyclopropenoid acids appears to be greatly affected by operating conditions, interallia by level of liquid phase on the support, size of the injected sample, and column temperature in the range of 170-220°C. Cyclopropenoid esters either readily rearrange or decompose on the column to give spurious peaks. Methyl linoleate and methyl malvalate are either unresolved or incompletely resolved on both polar and non-polar columns. If highly inert supports and silicon liquid phases are used, however, successful GLC of the native ester is possible. Recourt *et al.*²⁷ have successfully chromatographed methyl stercolate on a well conditioned EGSS-X column where it eluted just after methyl linoleate.

Very recently Fisher and Schuller²⁸ have developed a gas chromatographic method of analysis of cyclopropenoid acids in several oils that contained 8-58% CPFA, using a glass column packed with a methyl silicone substrate on an inert support.

To overcome this difficulty for analytical purposes, the cyclopropenoid components are suitably modified chemically by hydrogenation^{29,30,31}, or by reaction with mercaptans^{8,18} or silver nitrate, in this way the analysis is quantitative.

1) Hydrogenation

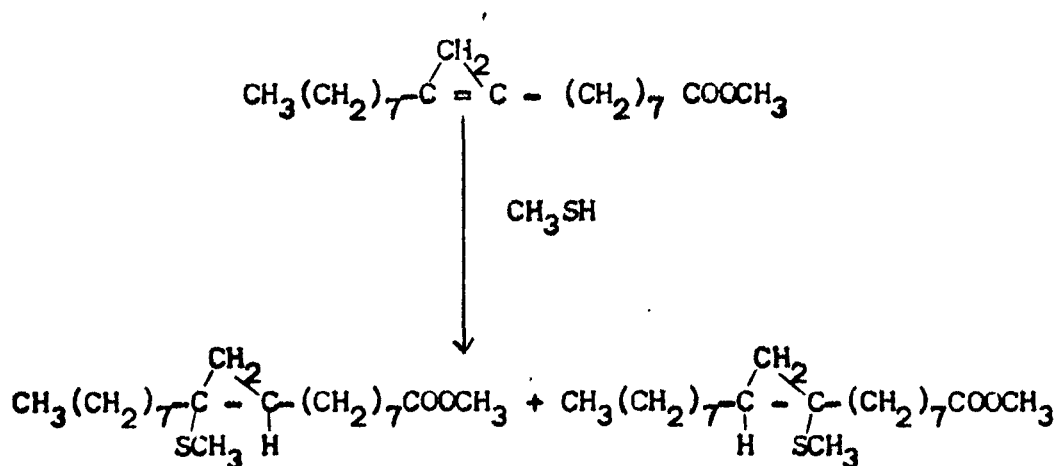
Individual cyclopropenoid acid (as their methyl esters) in a seed oil can be estimated by GLC analysis of the stable cyclopropane and branched-chain esters obtained by hydrogenation²⁹, after a preliminary analysis of the unmodified mixture to determine other unsaturated components. Miwa³⁰ has reviewed the complexity of such systems particularly when malvalic acid co-occurs with large amount of sterculic acid. Gellerman *et al.*³¹ reported that Lindlar's catalyst (partially poisoned palladium) which is commonly used to reduce acetylenes to olefins, reduces cyclopropenes to cyclopropanes with no over-reduction and without affecting any normal olefinic compounds which may be present. The resulting cyclopropane acid can be isolated, if necessary, for its structure determination by appropriate technique.

ii) GLC of methyl mercaptan derivatives

The hydrogenation process often gives rise to a number of side products, unless conducted under very closely controlled conditions. In addition, it does not allow the analysis of the other unsaturated fatty acids. The addition reaction of mercaptan to cyclopropene compounds by Kircher¹⁸ has suggested a new approach to the analysis of these compounds. Raju and Reiser⁸ utilised this reaction for the quantitative

estimation of CPFA as their methyl mercaptan derivatives. This method estimates individual CPFA as well as normal and cyclopropane acids.

Mercaptans add readily across the cyclic double bonds in a CPFA to give two unresolved isomeric thiol esters. The exact nature of the reaction is not known. They can undergo either a free radical or nucleophilic addition and the reaction may be represented as follows :

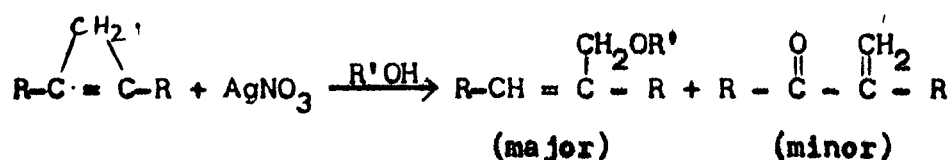


The absence of any additional peaks on gas chromatogram indicates that no side reaction occurs. However, if the reaction is carried out above 35°C, the methyl linoleate forms an additional product which will give a peak in between the malvalate and sterculate derivatives. Other fatty acids including any cyclopropane components are not affected by the reagent and can be estimated simultaneously from the same chromatogram.

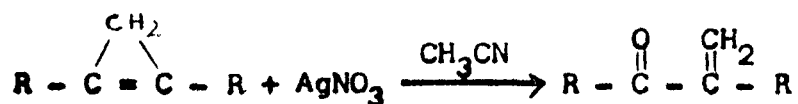
The methanethiol adducts showed no sign of decomposition on GLC column up to 240°C and have longer retention times than normal fatty esters due to the presence of -SCH₃ group, so that individual cyclopropenoid components can be separated and estimated by this procedure.

iii) GLC of silver nitrate derivative

In 1965, Kircher³² investigated the reaction of storculene (1,2-di-n-octylcyclopropene) with AgNO₃. The reaction is rapid in alcohols and the product is largely an alkoxymethyl olefin plus smaller amount of an α,β -unsaturated ketone.



In monohydroxylic solvents (such as acetonitrile, acetone), the reaction is slower and an α,β -unsaturated ketone is the only product observed.



Kircher showed the utility of this reaction for the qualitative analysis of the CPFA in S. foetida oil and proposed that this reaction be utilized for the quantitative analysis of cyclopropenoid acids in seed oils. Cyclopropane or other unsaturated

components present initially are unaffected and can be identified and determined separately.

Recently, Schneider et al.³³ reported that this method is applicable to oils containing from 0.01% to 100% of cyclopropenoid fatty acids. The derivatives of oils containing low levels of cyclopropenoids are separated from the normal methyl esters by alumina chromatography prior to GLC. When analysing oils that contain low levels of cyclopropenoid (< 5.0%), the normal methyl esters and the reaction products of cyclopropenoid must be separated by alumina chromatography to prevent overloading of the GLC column.

IV) Spectroscopic Techniques

Cyclopropenoid fatty acids in seed oils can often be detected or identified spectroscopically.

a) Infrared Spectrometry

Cyclopropenoid fatty acids showed two distinctive prominent bands in the IR spectrum, one at $1008-1010\text{ cm}^{-1}$ is attributed to the in-plane wagging vibration of the ring methylene group; the additional weaker band at 1852 cm^{-1} is probably the stretching frequency of the ring double bond and can be demonstrated when purified compounds or fractions are available but are of lesser value in the examination of crude

oils. Measurement of the absorption at 1010 cm^{-1} has been suggested as a means of estimating the total cyclopropenoid content of a seed oil.

b) Nuclear Magnetic Resonance Spectrometry

In recent years, NMR spectroscopy has been increasingly applied to the identification of lipid structures and is a rapid, simple method of analysis for the cyclopropene function in the lipids. Cyclopropene ring methylene protons give rise to distinctive signal at 9.2τ (singlet).

c) Mass Spectrometry

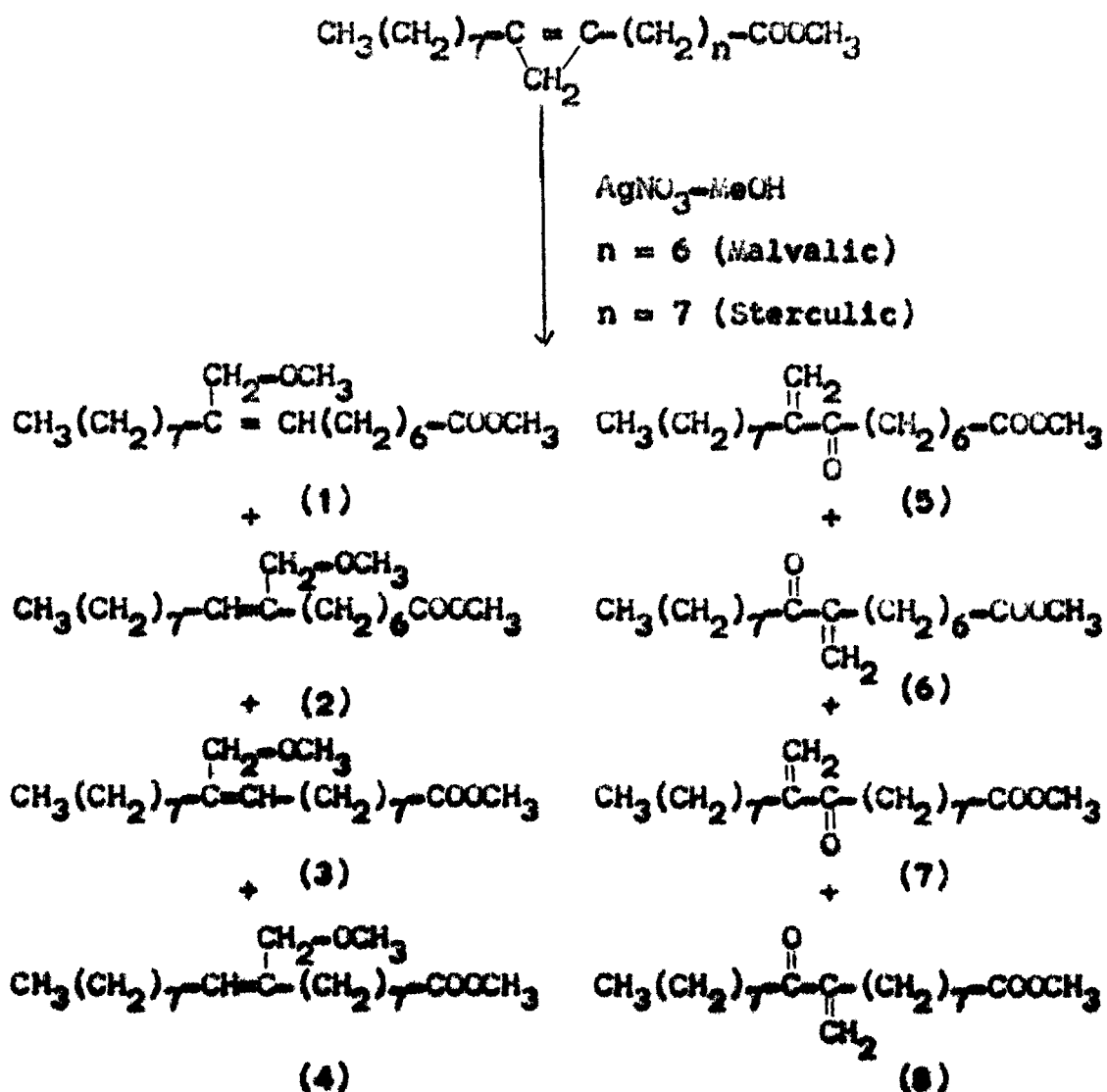
Like the unsaturated compounds cyclopropene acids are very unstable under electron bombardment and they isomerise to a mixture of isomeric alkanoates. The cyclopropene system may be fixed by some chemical reaction leading to a product which are more usually a mixture of products identifiable by mass spectrometry. These reactions include

- i) Silver nitrate methanol treated derivatives,
- ii) Diketo derivatives,
- iii) Thiol derivatives, and
- iv) Catalytic hydrogenation.

i) AgNO₃-MeOH treated derivatives

Several workers^{13,34,35} have carried out mass spectra of AgNO₃-MeOH treated derivatives of cyclopropenoid fatty acids (CPFA) to locate the position of cyclopropene group.

Cyclopropenoid fatty acids (Malvalic and Sterculic) react with AgNO₃-MeOH to give the following isomeric ether and keto derivatives as shown in the Scheme-1.



Scheme-1

1) MS of ether derivatives of Methyl Malvalate and Methyl Sterculate

The mass spectrum of the ether derivatives (1 and 2) (Fig. 1) showed molecular ion peak at m/z 326 ($C_{20}H_{38}O_3$) followed by other significant peaks at m/z 295 ($M-OCH_3$), 294 ($M-CH_3OH$), 279, 213, 197, 196, 195, 193, 183, 182, 181, 179, 166, 165, 164, 163, 152, 151, 150, 149, 140, 137, 136, 135, 124, 123, 122, 121, 119, 111 and 110 (the lower mass peaks are unrecorded) and the MS of (3 and 4) (Fig. 2) gave molecular ion peak at m/z 340 ($C_{21}H_{46}O_3$) with other significant peaks at 309 ($M-OCH_3$), 308 ($M-CH_3OH$), 277 ($M-31-32$), 241, 227 ($M-113$), 210, 209, 197, 195, 193, 183, 179, 166, 165, 164, 163, 154, 152, 150, 149, 137, 136, 135, 125, 124, 123, 122, 121, 111 and 110; lower mass peaks were not recorded.

Peaks which are of greater interest for diagnostic purposes are those ions which indicate the position of cyclopropene ring.

Isomers (1 and 3) have similar fragmentation pattern.

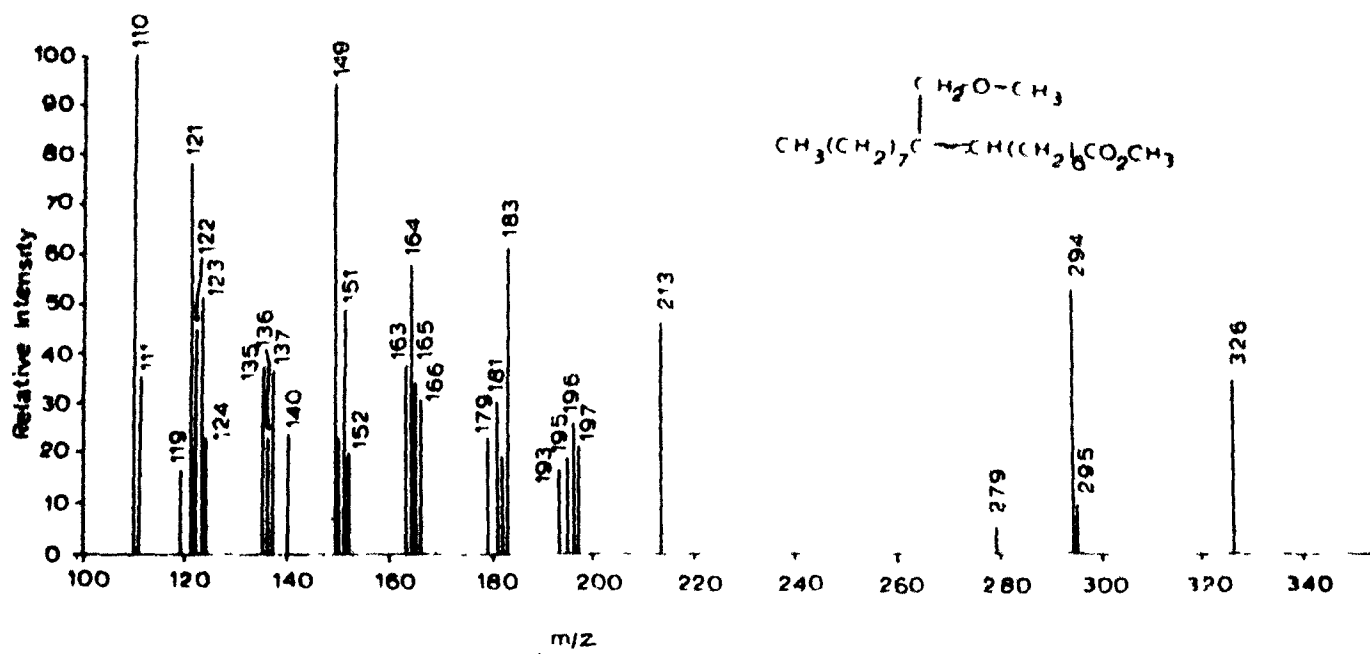


Fig1 MASS SPECTRUM OF (1)

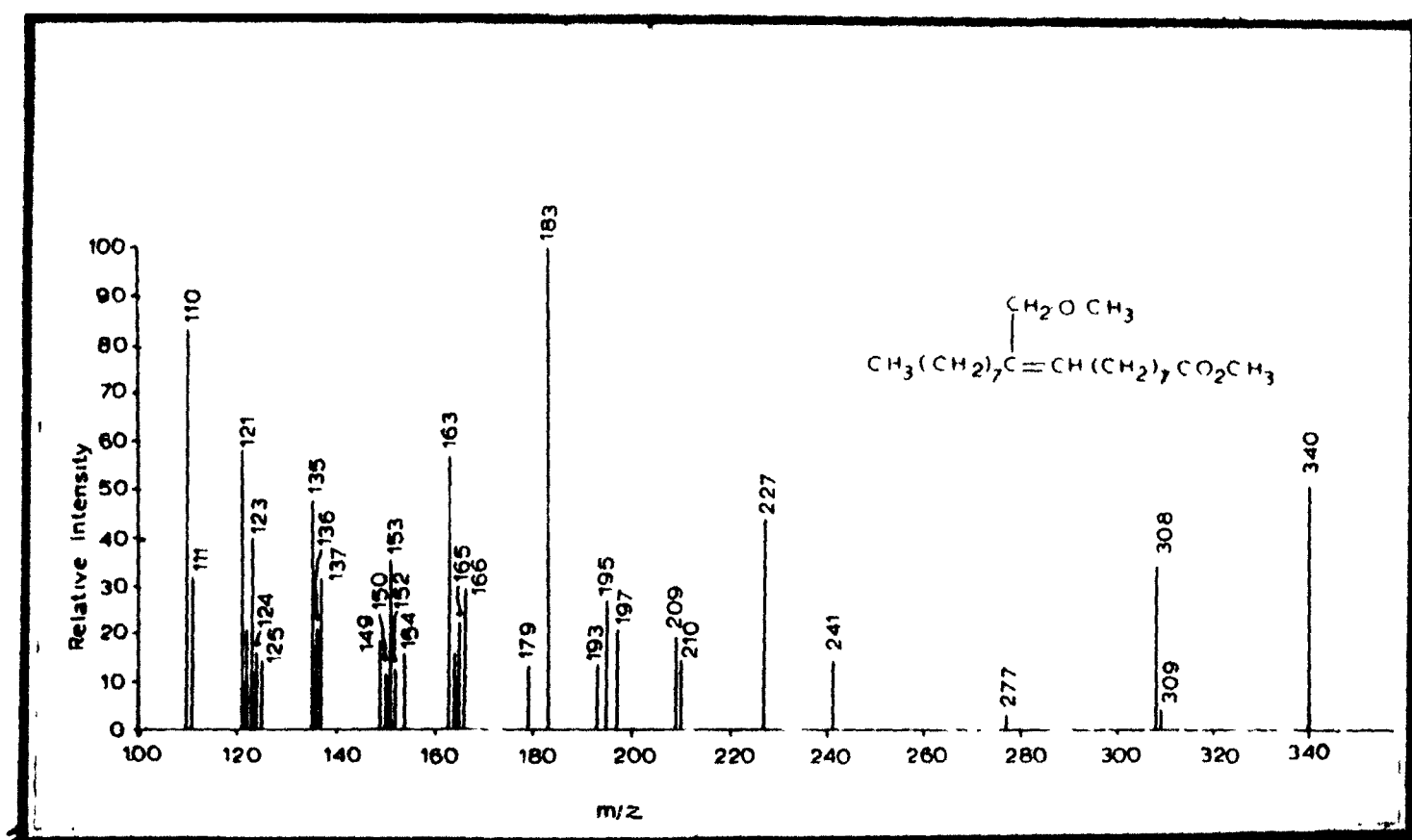
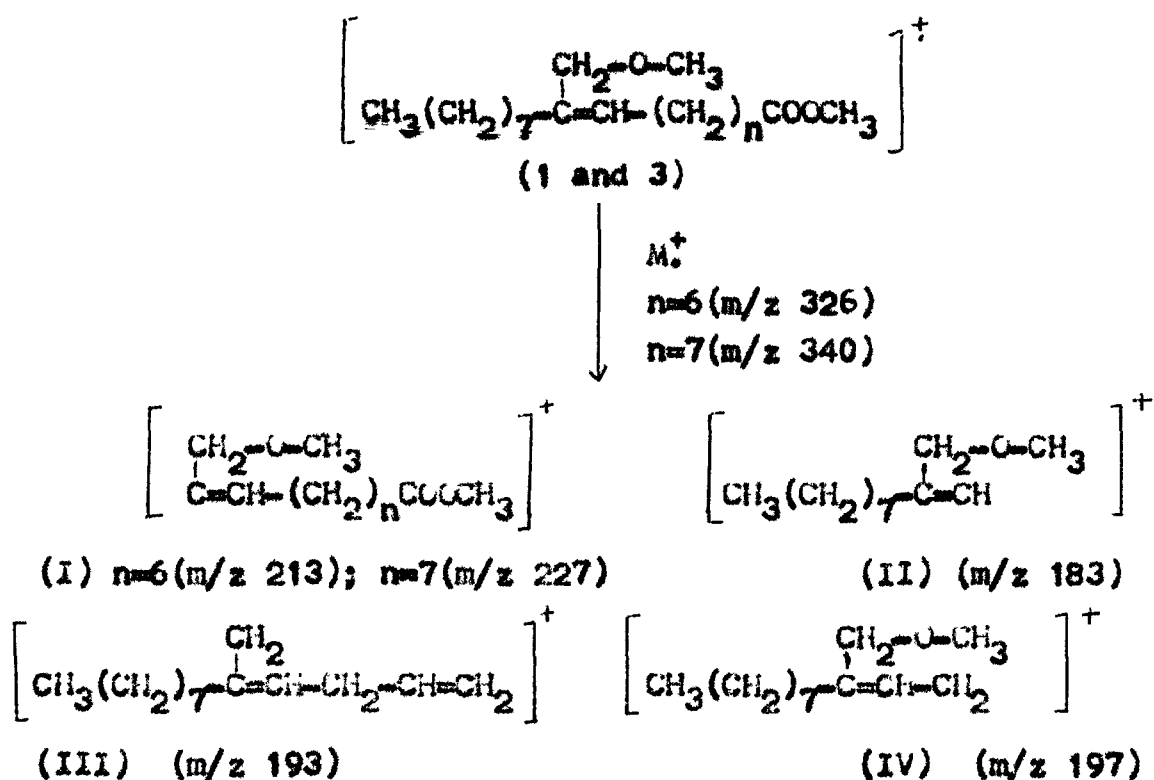
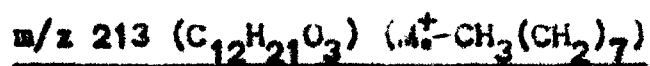


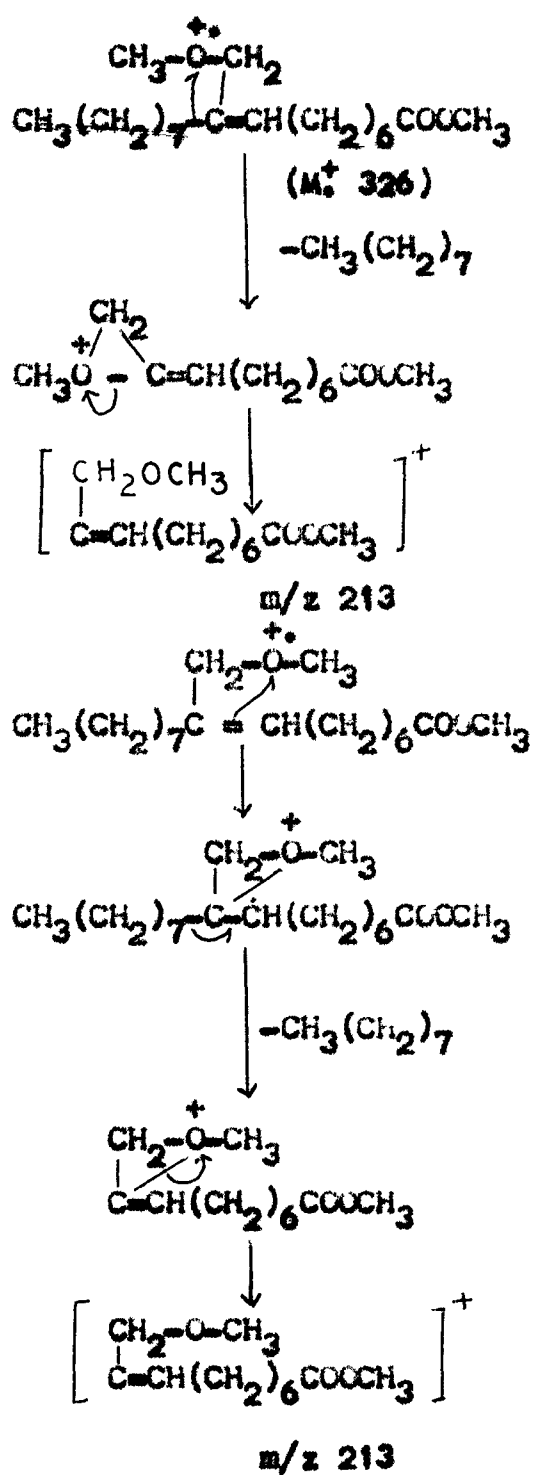
Fig 2. MASS SPECTRUM OF (3)



Ions I-IV are important components in each spectrum. The formation of these ions can be shown as follows.

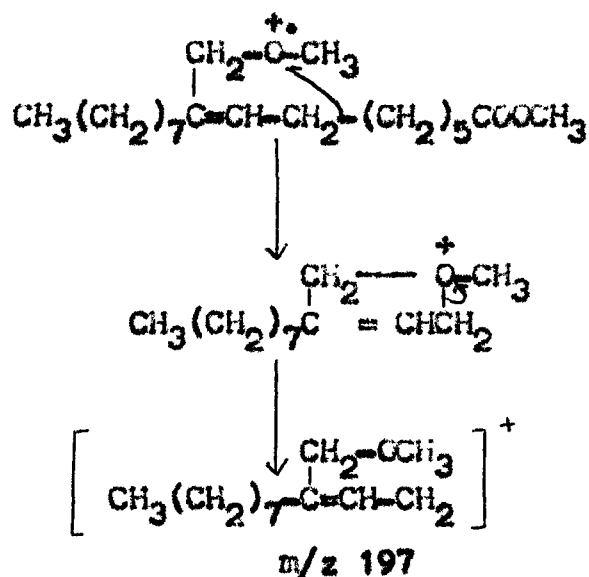


This fragment ion which agrees with the loss of mass unit 113 from the molecular ion can be rationalised in two ways as shown in scheme below.

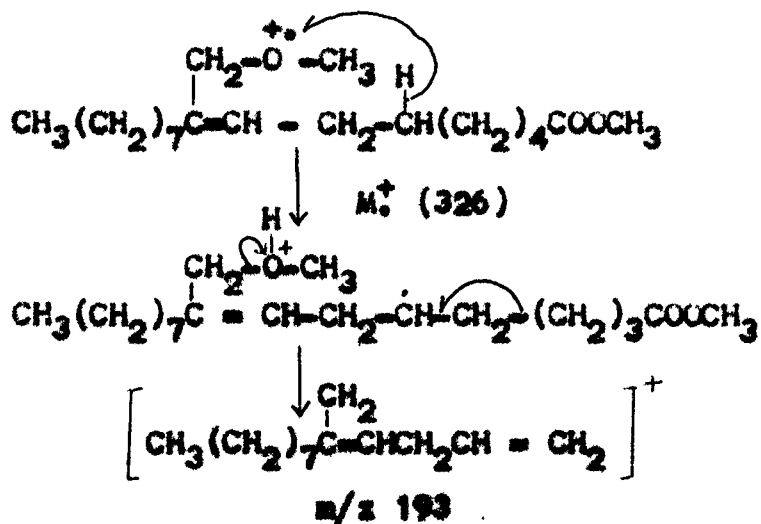


m/z 197 (C₁₃H₂₅O) (M⁺-129)

The composition of this ion, C₁₃H₂₅O, suggested that it originated by the loss of C₇H₁₃O₂ from the molecular ion as shown below.

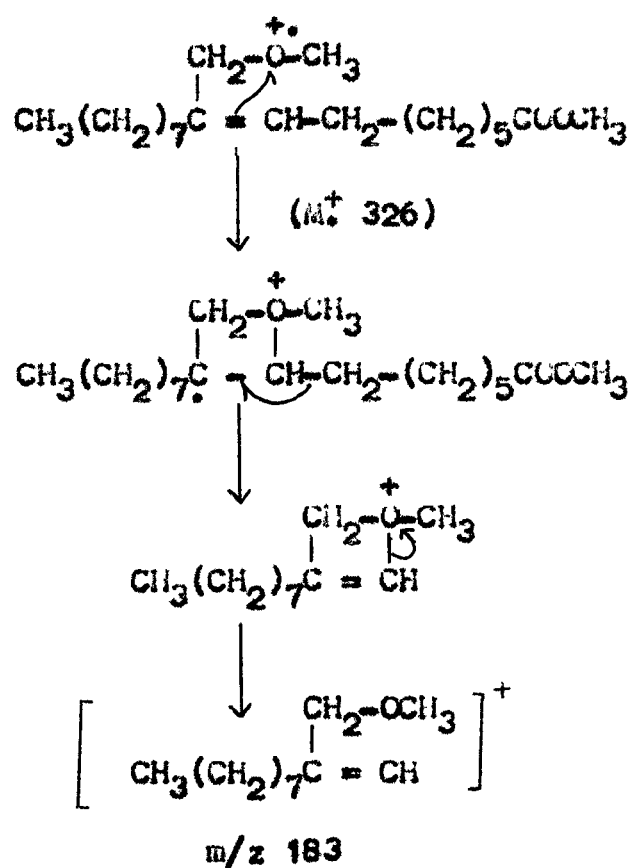
m/z 193 (C₁₄H₂₅)

This fragment ion seems to have been formed by the loss of mass unit 133 from the molecular ion as shown below.

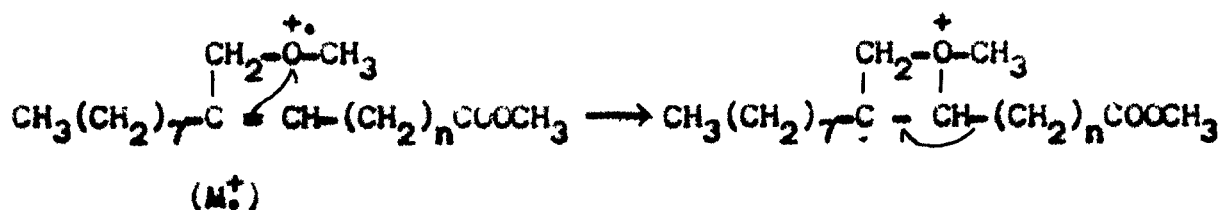


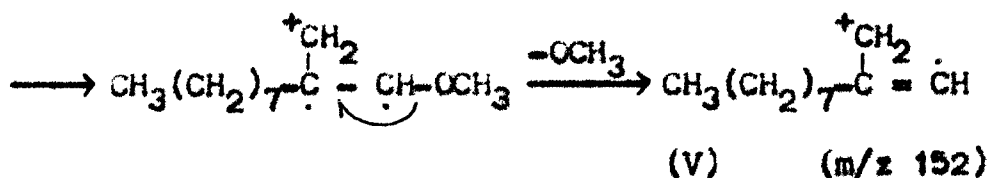
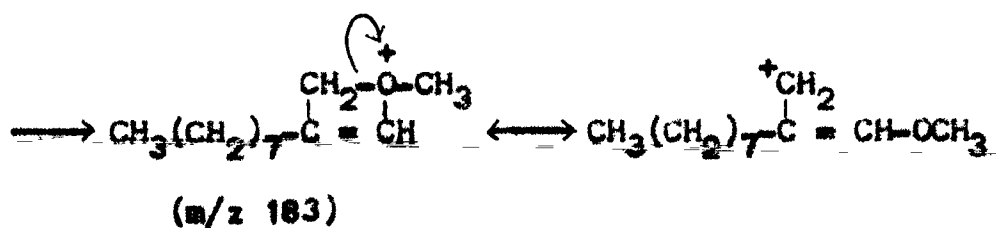
m/z 183 (C₁₂H₂₂O)

The formation of this fragment ion has been rationalised according to the following scheme.

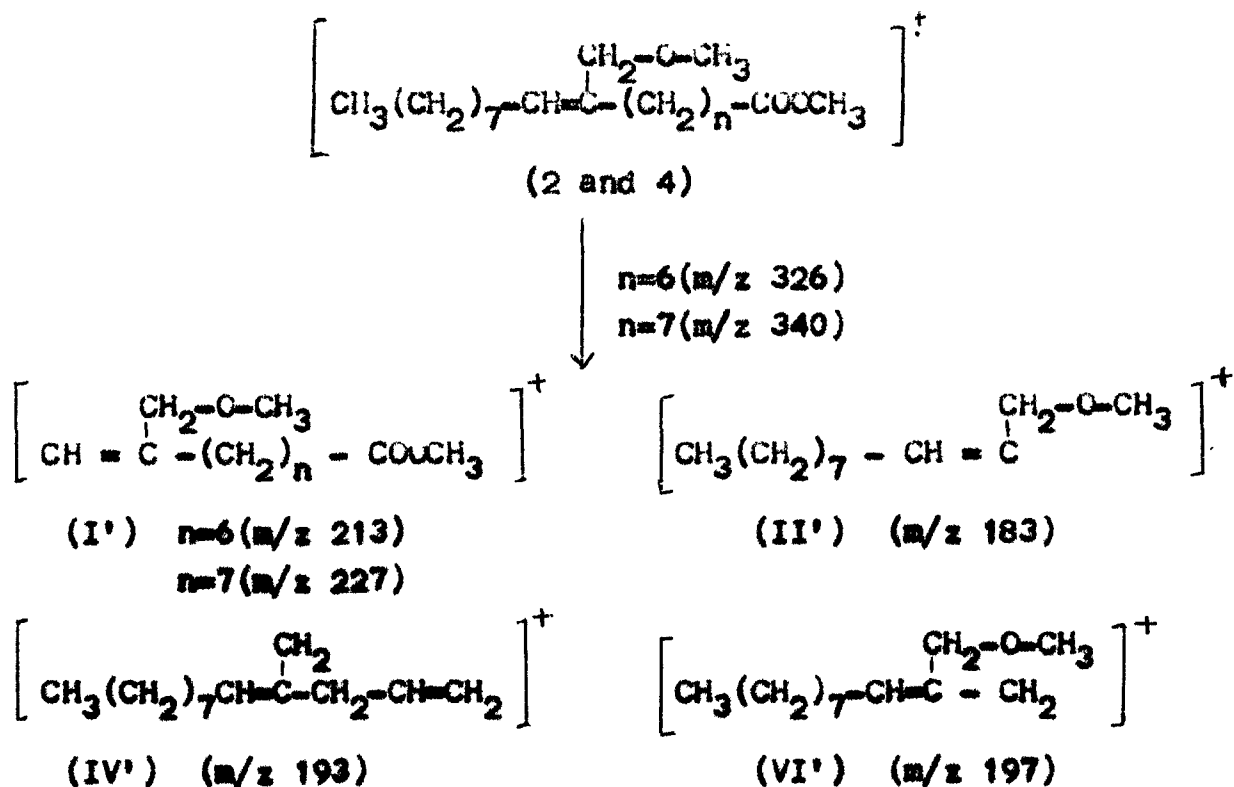


A characteristic peak at m/z 152 is the ion V, i.e. 183-31 resulting from cleavage with respect to the side chain ether group.

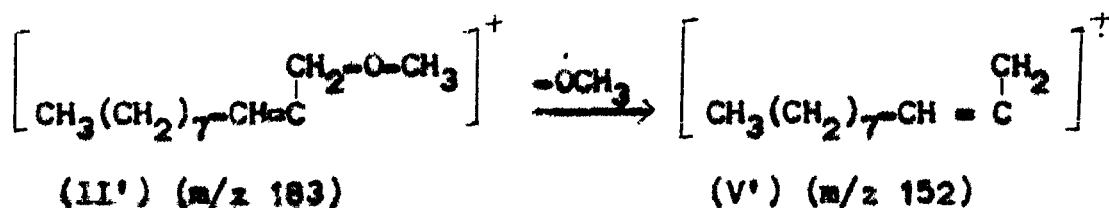




The fragmentation pattern and the molecular ion of isomers (2 and 4) are also very similar.



The peak of m/z 152 (V') can also be shown to arise for these two isomers.



There are few fragment ion peaks in the spectra of the ether derivatives (1 and 3) and for isomeric (2 and 4) which need some comment. The mass ion peaks with m/z 210 and 209 in the spectrum of (3 and 4) could be considered as analogous to the mass ion peaks with m/z 196 and 195 in the spectrum (1 and 2). Interestingly no analogous peak of m/z 197 of (1 and 2) is observed at m/z 211 in the case of (3 and 4). The mass ions 196 and 195 have been suggested to arise from the fragment ion m/z 197. Such a mechanism for the formation of mass ions 210 and 209 in the spectrum of (3 and 4) can not be shown in the absence of mass ion 211. This therefore, has necessitated the suggestion that the mass ions 210 and 209 are obtained by different pathways other than those shown for the genesis of mass ions 196 and 195 respectively.

11) MS of ketone derivatives

Cleavage on either side of the keto group in ketone derivatives (5-8) supported the position of cyclopropene ring in the fatty ester chain. The 8-keto derivatives(5) (Fig.-3) showed a weak but significant molecular ion peak of m/z 310. The diagnostic ion peak at m/z 143 (VI), which arises by the preferred cleavage \propto to the keto group, is alone sufficient to

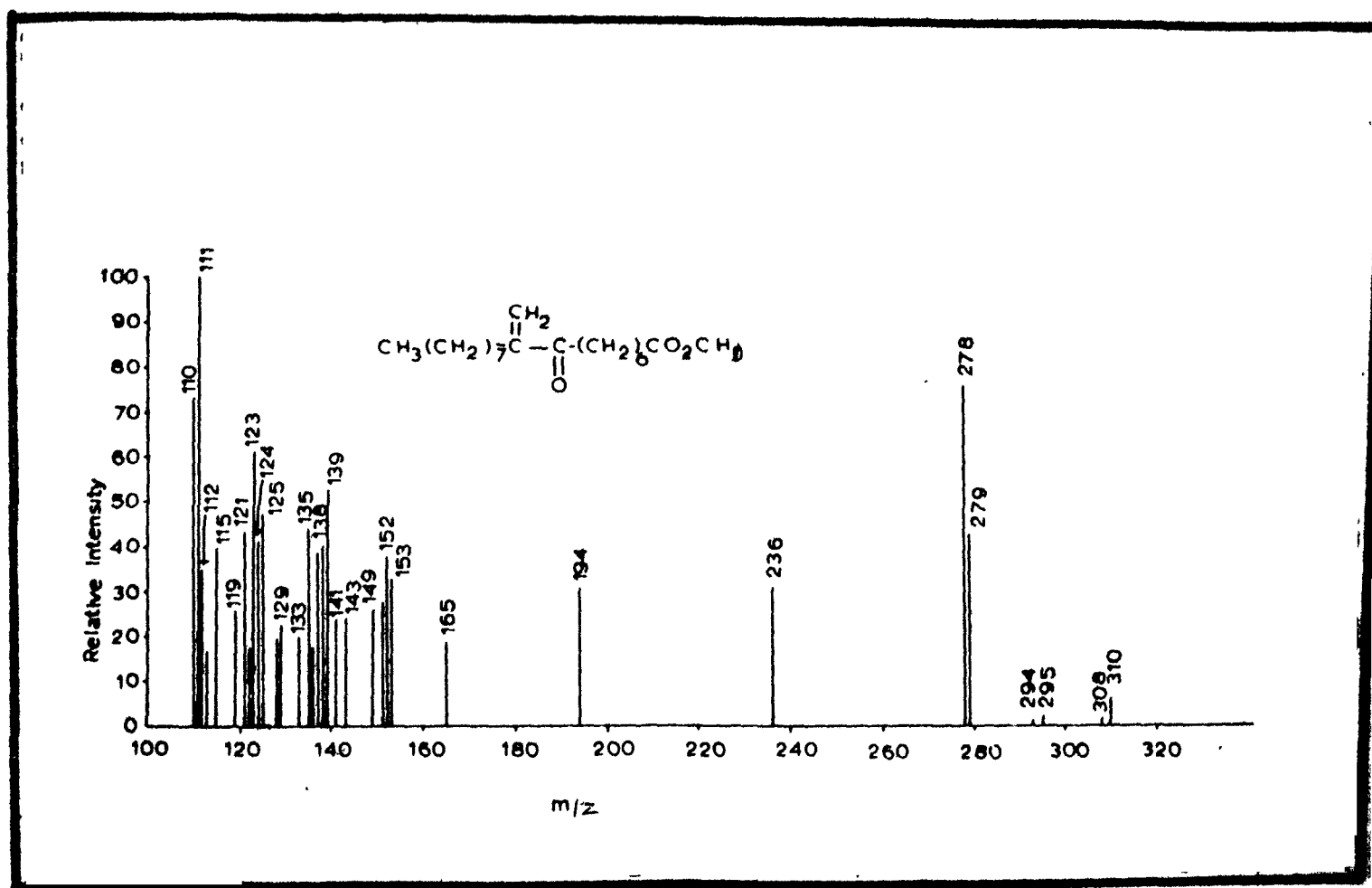
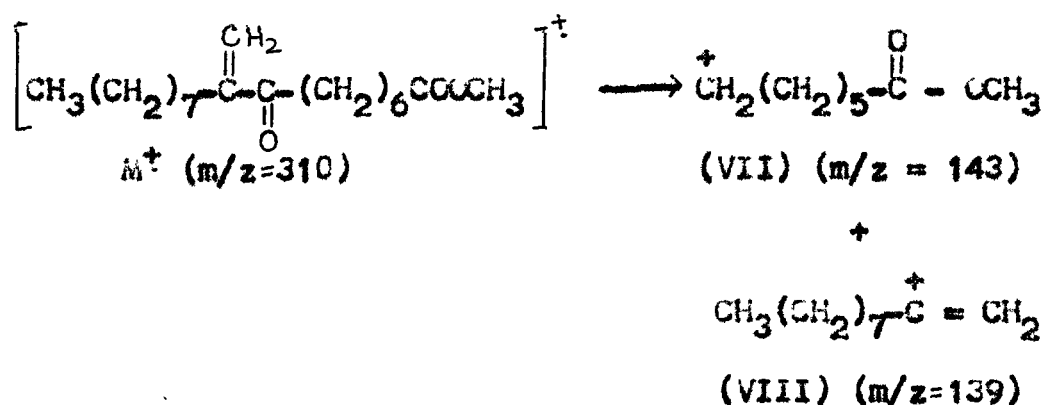
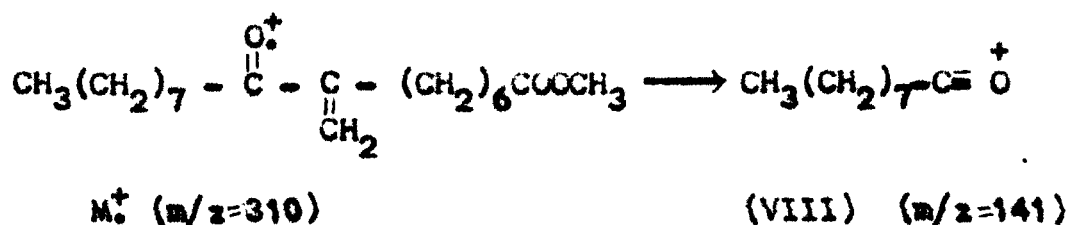


Fig. 3. MASS SPECTRUM OF (5)

locate the three membered cyclopropene ring on the chain at 8,9-position. The fragment companion peak at m/z 139 (VII), corresponding to cleavage on the other side of the keto group, support the assignment.



The isomeric ketone (6) also supports that the cyclopropene ring is between C_8 and C_9 position. The diagnostic ion peak at m/z 141 (VIII) corresponding to the cleavage between the α, β -unsaturated ketone.



In the mass spectrum of the 9-keto derivative (7) (Fig.-4) (M^+ 324), the most intense peak at m/z 167 (IX), corresponding to one of the fragment of α -keto cleavage, again immediately and correctly establish the positions at cyclopropene ring at

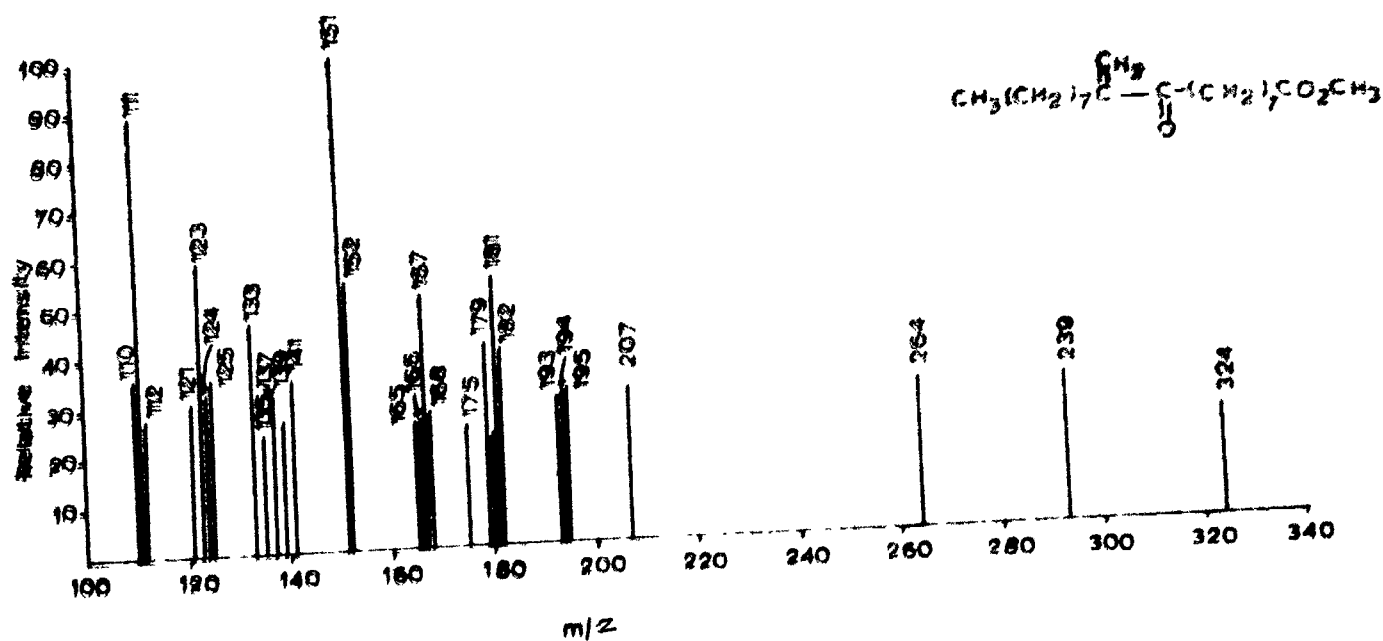
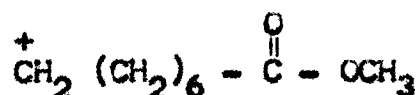
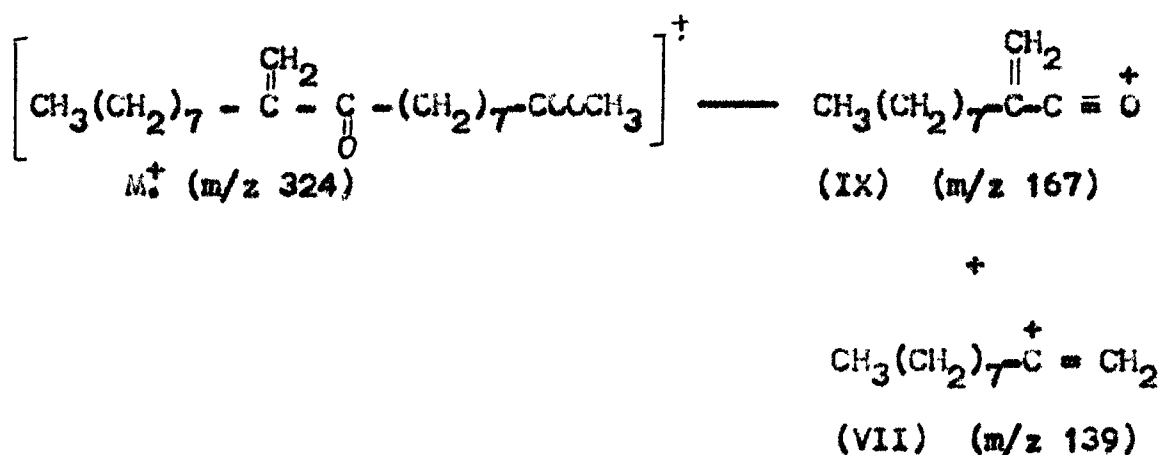


Fig 4. MASS SPECTRUM OF (7)

the 9,10-position. The other product of α -keto cleavage, m/z 157 :



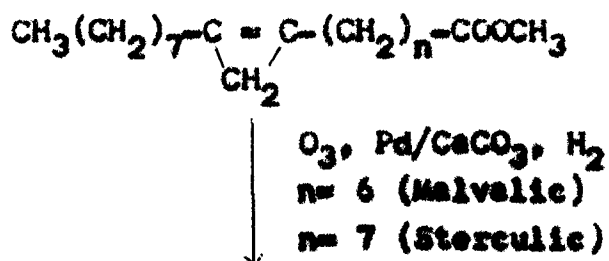
does not appear and therefore is not used for the assignment.

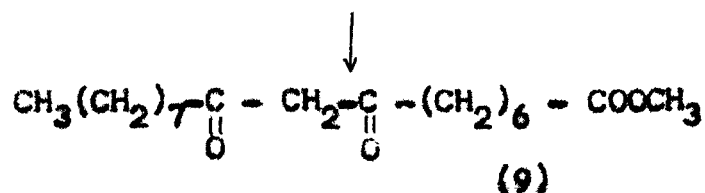


The appearance of peak at m/z 141 (VIII) in the isomeric ketone (8) further supported that the position of cyclopropene ring is between $\text{C}_{9,10}$ -position.

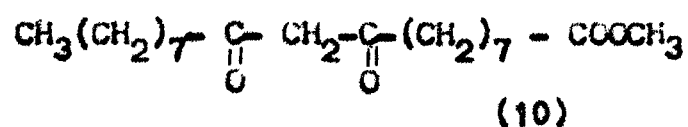
ii) Diketo Derivatives

Cyclopropenoid fatty acids (malvalic and sterculic) on ozonolysis give diketo derivatives as given below.





+



The 1,3-diketone system produced by oxidative cleavage of the ring double bond provides increased stability and polarity and can be identified by a color reaction resulting from treatment with ferric ion.³ The diketo acids are crystalline solids with intense absorption at 276 mμ. The 18-carbon compound had a melting point of 56.0-56.2°C, while the 19-carbon compound melted at 58.0-58.2°C. The esters of various chain lengths can be separated by gas-liquid or reversed phase chromatography, and mass spectra of the esters provide an excellent method for locating the position of the ring⁶.

The MS of diketo derivatives (9 and 10) (Figs. 5 & 6) showed molecular ion peaks (M) at m/z 326 and 340 respectively followed by other significant peaks at m/z M-31, M-98, M-113, 211, 198, 183, M-145, M-155, M-167, 141, 113.

The prominent fragment ions which locate the position of cyclopropene ring are shown below :

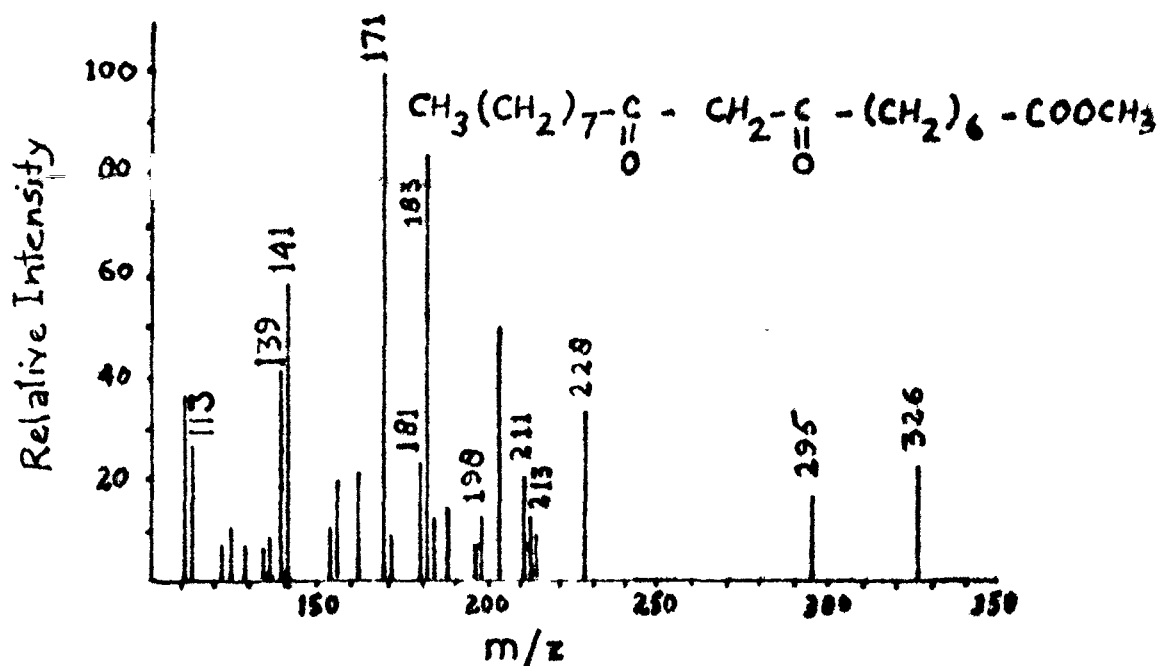


Fig 5. MASS SPECTRUM OF (9)

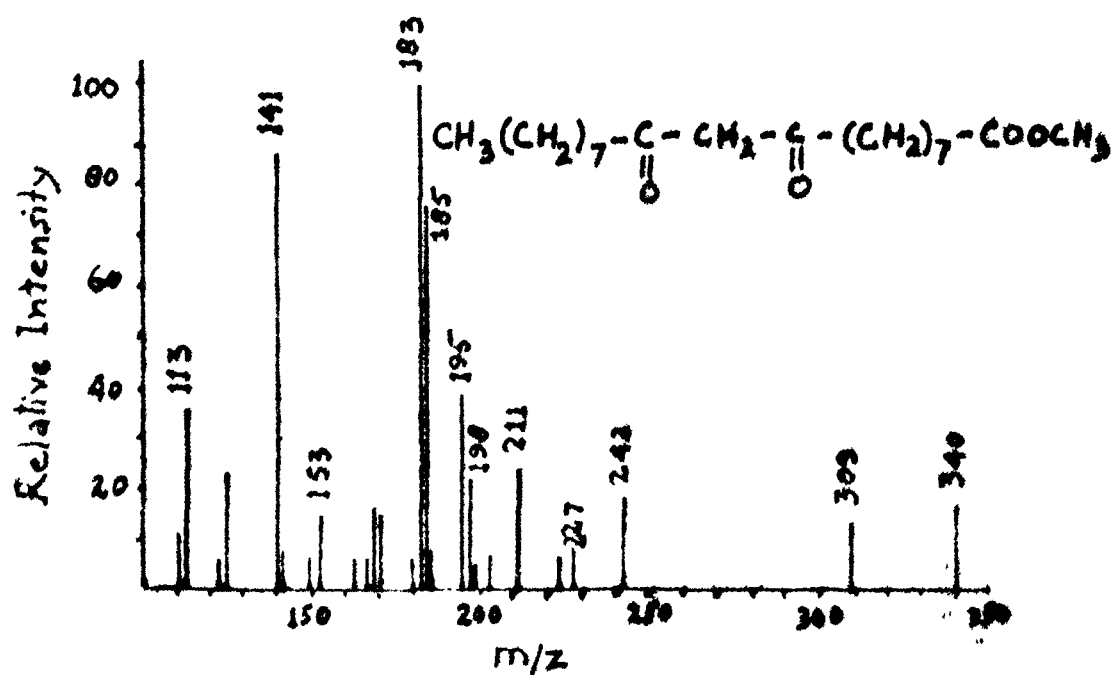
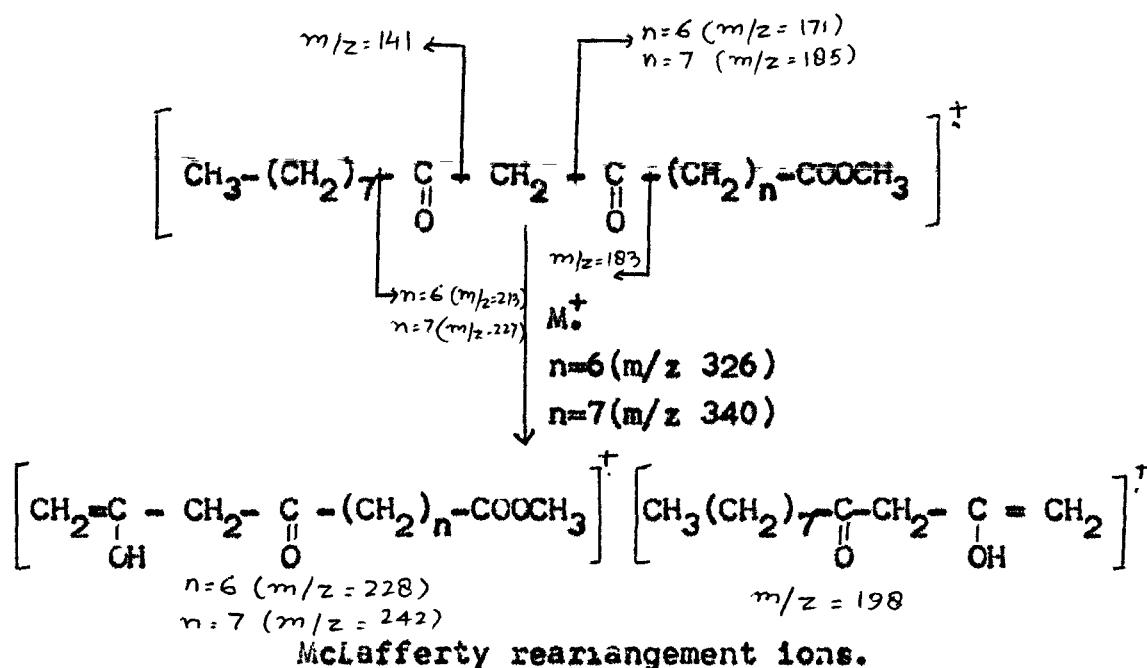
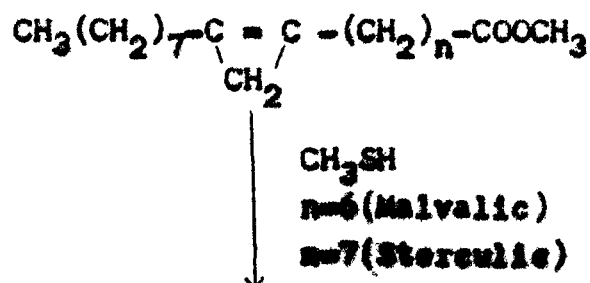


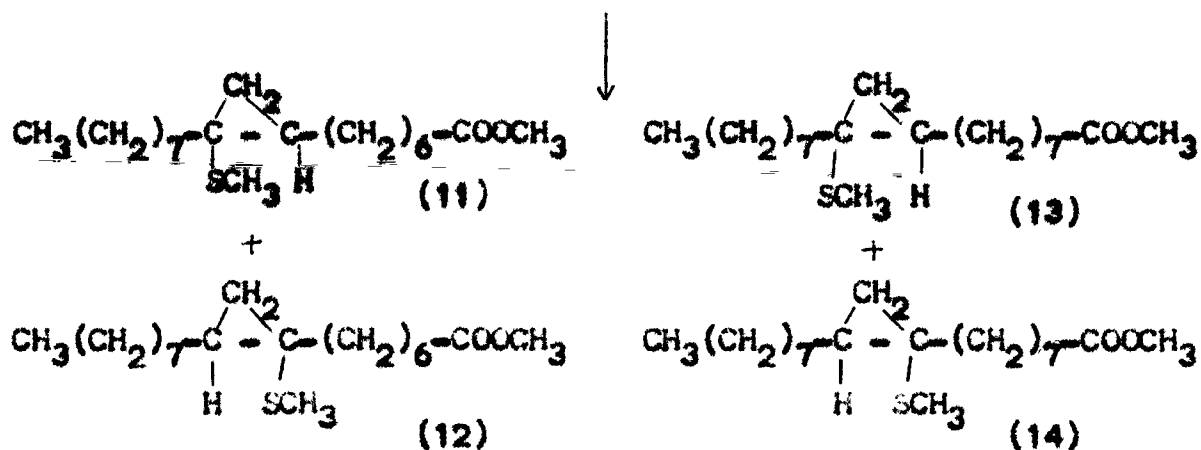
Fig 6. MASS SPECTRUM OF (10)



iii) Thiol Derivatives

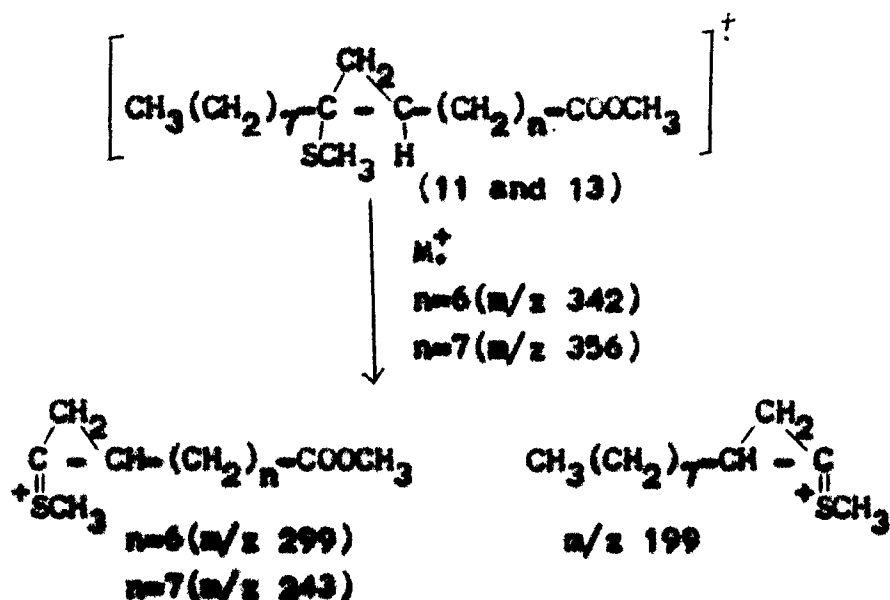
The addition of methanethiol across the ring double bond has been employed by Raju and Reiser⁸ for the preparation of distinctive derivatives useful in gas chromatographic separations. The technique is simple and quantitative. The derivatives appear to have high stability. The thiol is added randomly to the double bond to give an unresolved mixture of isomers in which the sulphur atom is attached at one or the other end of the original double bond. The mass spectrometric examination of the mixture of thiol addition derivatives makes a definite assignment of the ring position possible.





The mass spectrum of thiol derivatives of (11 and 12) (Fig. 7) and (13 and 14) (Fig. 8) gave molecular ion peaks (M) at m/z 342 and 356 respectively followed by other significant peaks at m/z $M-15$, $M-31$, $M-47$, $M-79$, $M-97$, $M-113$, 199.

Fragment ions which indicate the position of the cyclopropene ring are important. Fragmentation pattern and molecular ions of isomers (11 and 13) are similar. The prominent fragment ions which locate the position of cyclopropene ring are given below.



(12 and 14) will show same fragmentation pattern.

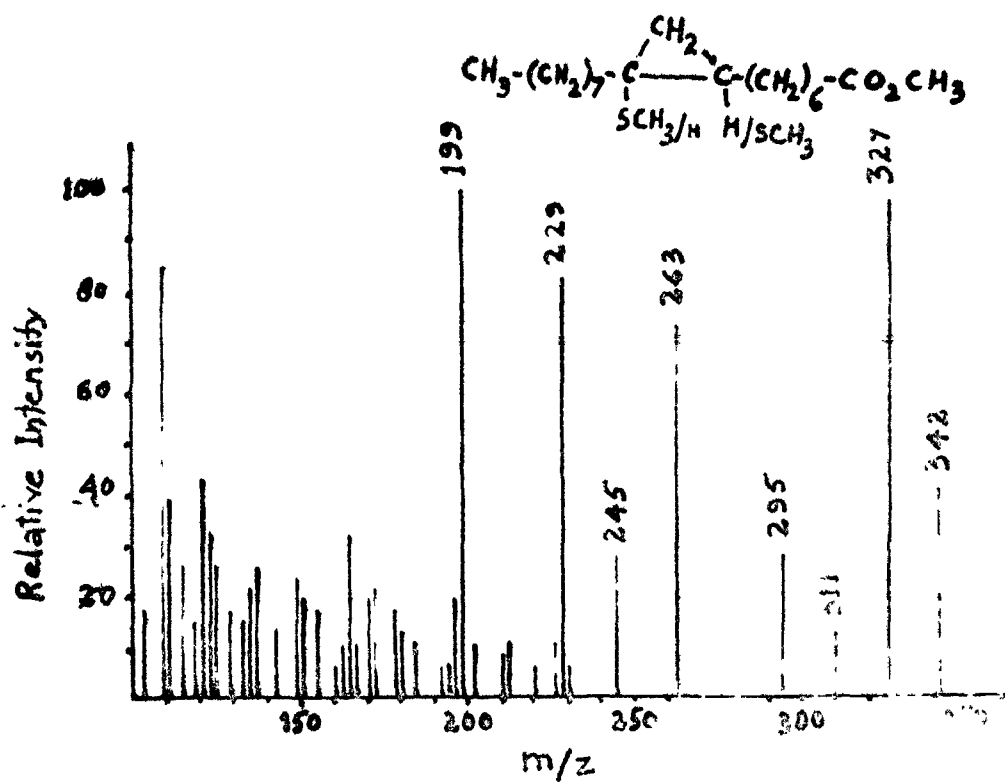


Fig 7. MASS SPECTRUM OF (11) (12)

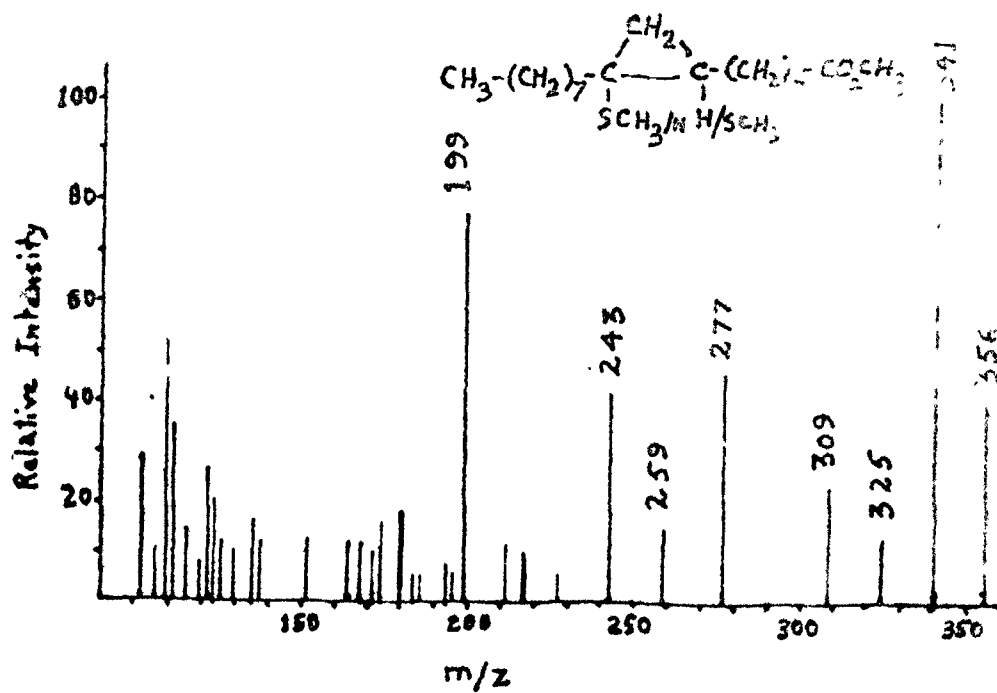
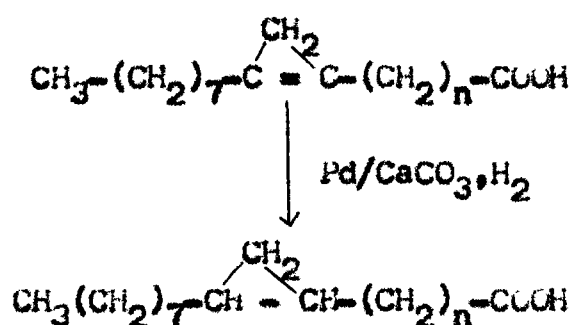


Fig 8. MASS SPECTRUM OF (13) (14)

iv) Catalytic Hydrogenation

Cyclopropene acids can be reduced to the much less reactive cyclopropane acids by catalytic hydrogenation in the presence of palladium catalyst in alcohol.³⁶



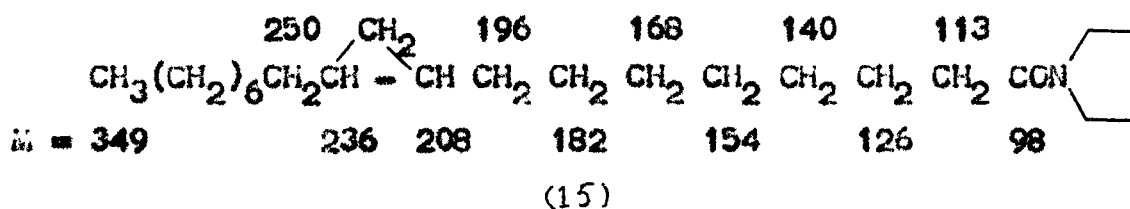
Further reduction to the ring-opened compounds³⁷ allows assignment of ring location by mass spectrometry but this process is more complicated than the previously described methods. Furthermore, the natural sources of cyclopropene acids generally yield the analogous cyclopropane acids as well, and separation of these prior to catalytic reduction of cyclopropenes was, until recently difficult³⁸.

The position of cyclopropane ring can be fixed by MS after converting to an stable derivative.

- i) Pyrrolidides derivatives.
- ii) Special procedure for location of cyclopropane ring.

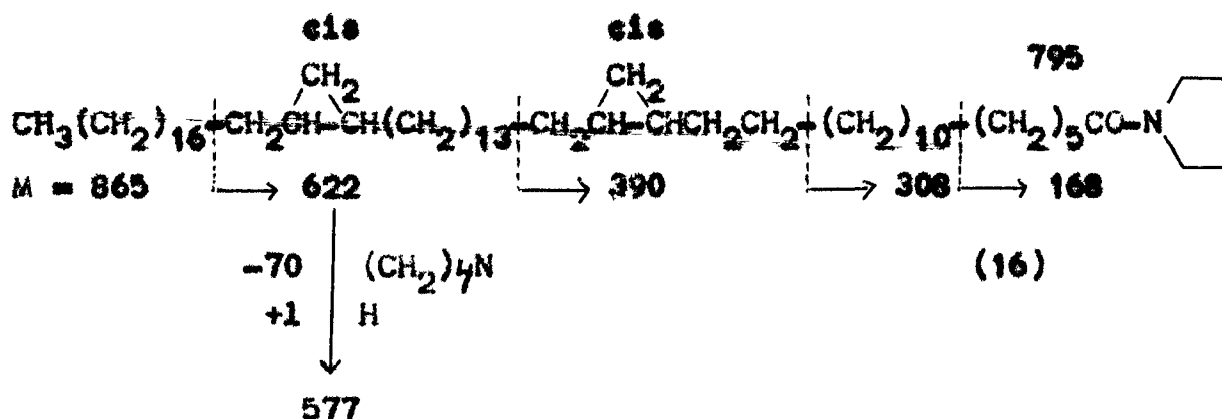
1) Pyrrolidides derivatives

Mass spectrometry of pyrrolidides has been investigated as a possible procedure for the location of cyclopropane rings by Gensler and Marshall³⁹. The mass spectrum of the pyrrolidide of cis-9,10-methylene octadecanoate (15) was not as readily interpreted as those of the simple monoenoic derivatives. The diagnostic fragments were not so even in intensity some being relatively more prominent (15) (low intensity peaks in brackets).



The most prominent ion in the homologous series was at m/z 250 representing cleavage to give a cyclopropylmethyl fragment, possibly stabilized by resonance with the cyclopropane ring. A difference of 12 mass units was found for the weak ions at m/z 196 and 208 which locate cyclopropane position and follows Andersson and Holman's rule⁴⁰ for the location of unsaturation also applied for this cyclopropane derivative.

The pyrrolidide of a synthetic bis cyclopropyl acid (16) related to degradation products from mycolic acids³⁹ did not contain clear sequences of cluster maxima in its mass spectrum leading to precise location of both cyclopropane rings.

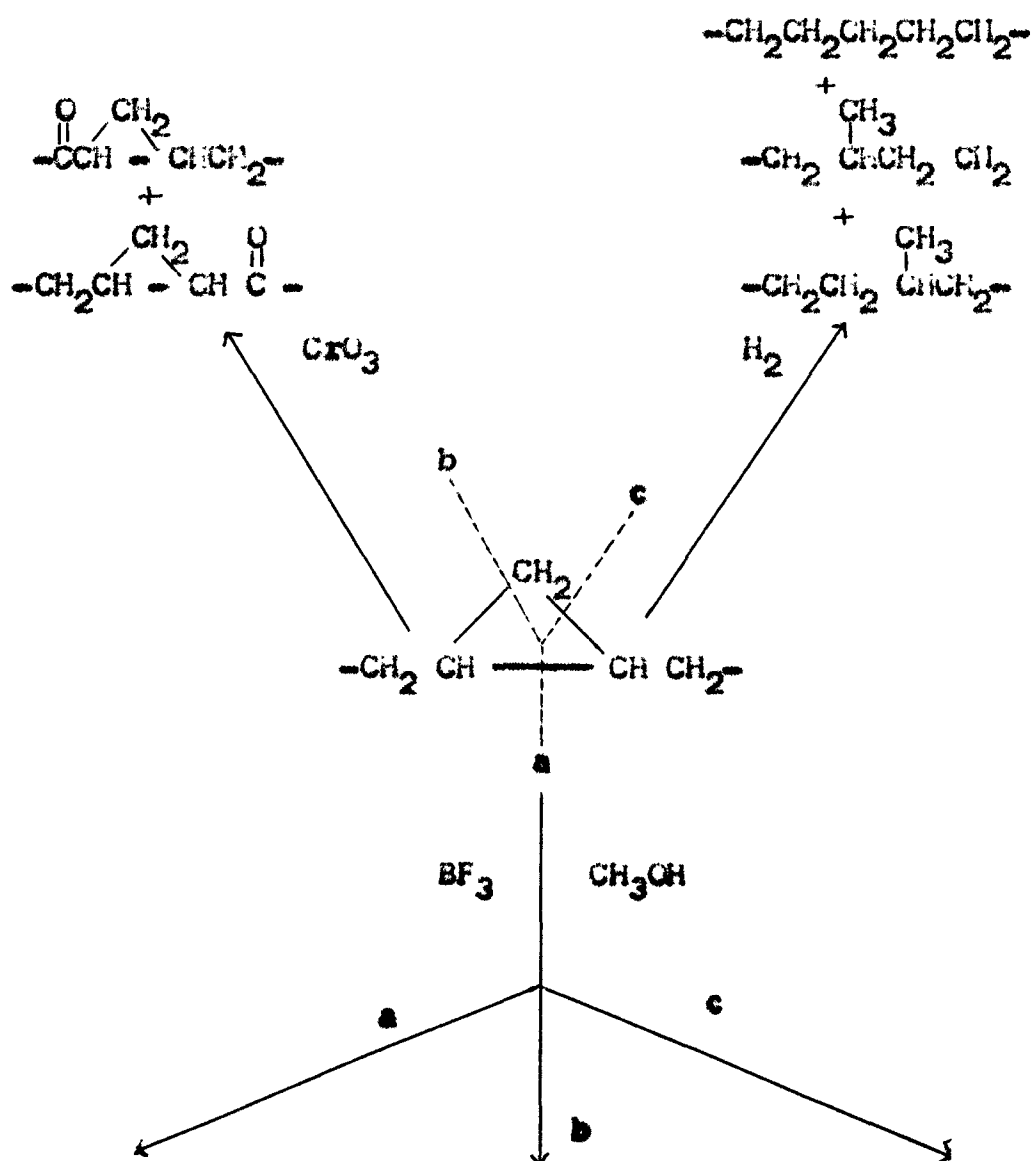


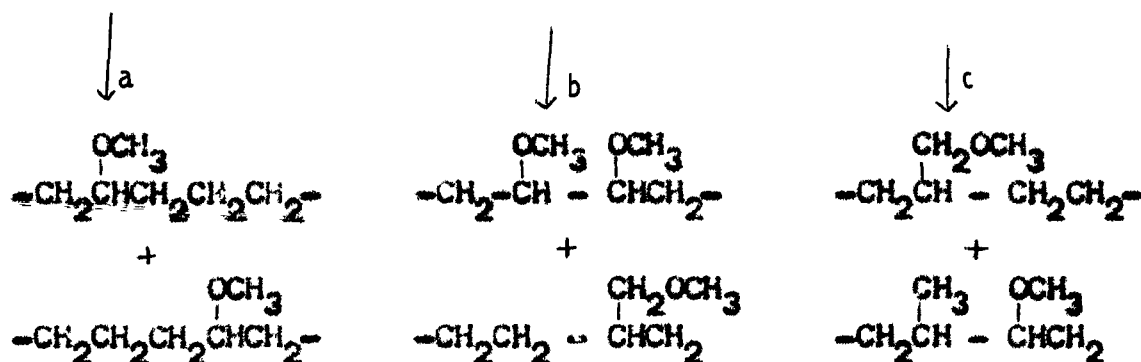
A difference of 12 mass units was found, for example, between m/z 364 and 276 which, according to the simple cyclopropane derivative (15), gave an erroneous assignment of one cyclopropane ring to the (21, 22) position. The mass spectrum did contain diagnostic fragments (16), however, two of which (m/z 390 and 626) corresponded to cyclopropylmethyl fragments and allowed location of the cyclopropane rings. The larger of these two fragments (m/z 626) was related to a peak at m/z 577 (16). The mass spectrum of the pyrrolidides of meromycolic acids, prepared from the mycolic acids of Mycobacterium tuberculosis, also contained peaks analogous to m/z 390 and 626 (16) which helped to confirm the position of the cyclopropane ring⁴¹.

11) Special procedures for location of cyclopropane rings

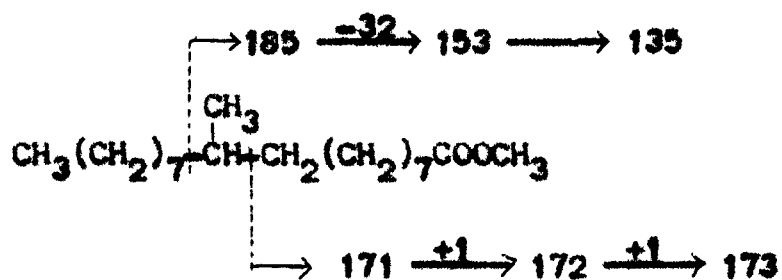
Although cyclopropane rings are attacked by many of the electrophilic reagents that attack double bonds, the reagents that form useful derivatives with the latter are not

suitable for the former. Peracids and Osmium tetroxide, for example, do not attack cyclopropane rings and although methoxymercuration of low molecular cyclopropanes is successful⁴² there are no reports of satisfactory reactions with long chain compounds. Three main approaches have been used to prepare derivatives suitable for mass spectrometric location of cyclopropane rings and these are summarised in the scheme (2).

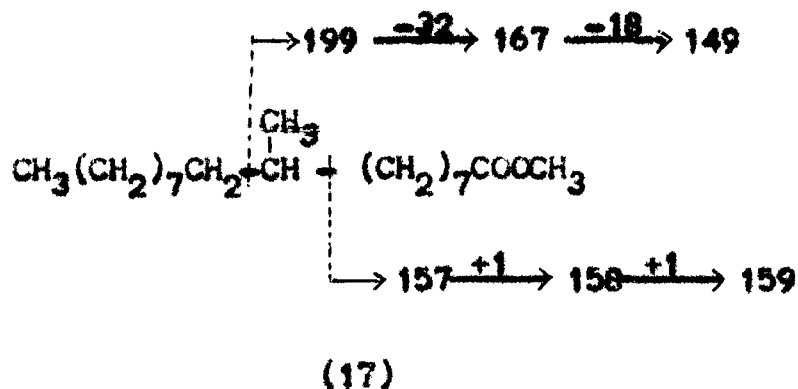


Scheme - 2

Cyclopropane esters having the ring beyond the (8,9) position, such as 9,10-methyleneoctadecanoate, gave rise to isomeric methyl branched esters (17) whose spectra contained two characteristic clusters of three low intensity peaks at m/z 157, 158, 159 and 171, 172, 173. Such a pattern, arising by cleavage at the point of branching and abstraction of one and two hydrogen atoms is characteristic of primary ions of this type^{43,44}. These characteristic clusters, separated by 14 mass units, are supported by peaks of enhanced intensity at m/z 185 and 199 and related peaks resulting from loss of elements of methanol and water (17).



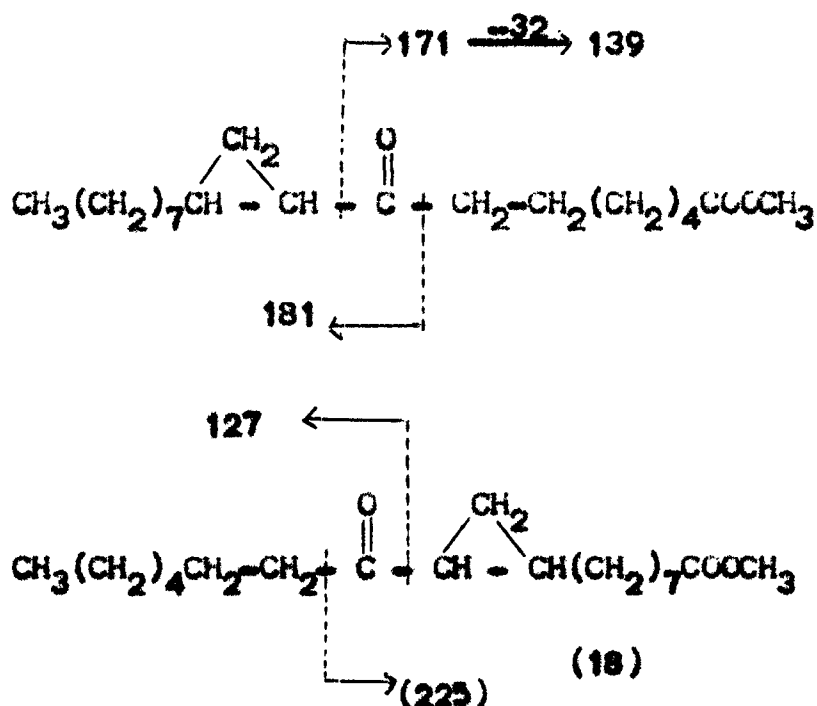
(17)



An 8-methyl branched ester results from hydrogenation of (7,8)- and (8,9)-cyclopropane esters and should contain in its mass spectrum a characteristic peak at m/z 143 corresponding to $\text{CH}_3\text{COO}(\text{CH}_2)_6^+$. This fragment, however is frequently very intense in long-chain esters so that careful inspection of the mass spectrum of the isomeric methyl branched esters is necessary for ring location in (7,8)- and (8,9)-cyclopropane acids. A valuable additional diagnostic fragment corresponding to $(M-76)^+$ is found in the mass spectrum of 6-methyl branched esters, so that (5,6)- and (6,7)-cyclopropane esters give isomeric methyl branched esters whose mass spectra contain these characteristic ions^{43,44}.

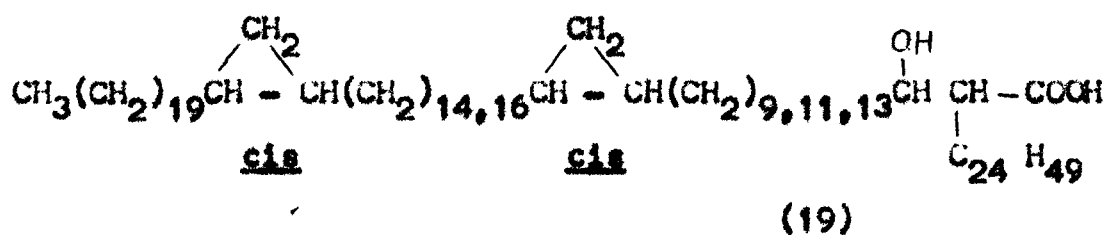
Two different approaches have been used successfully in locating cyclopropane rings in mycolic acids by mass spectrometry, namely the analysis of ketones prepared by chromium trioxide oxidation⁴⁵ and the use of derivatives resulting from BF_3 catalysed methoxylation⁴⁶.

Chromium trioxide oxidation of long-chain 1,2-disubstituted cyclopropane esters leads to isomeric keto esters such as those (18) resulting from oxidation of cis-9,10-methylenooctadecanoate. It proved possible to separate these isomeric keto esters (18) by TLC and study their mass spectra separately, but the derivatives from cis-6,7-methylenooctadecanoate were not resolved the position of the keto group in



the derivatives (18) was revealed by intense α -cleavages, supported by other characteristic less intense fragmentations. This procedure was applied to the acetate of a dicyclopropyl mycolic ester from Mycobacterium tuberculosis and led to the homologous structures indicated (19) for the parent mycolic

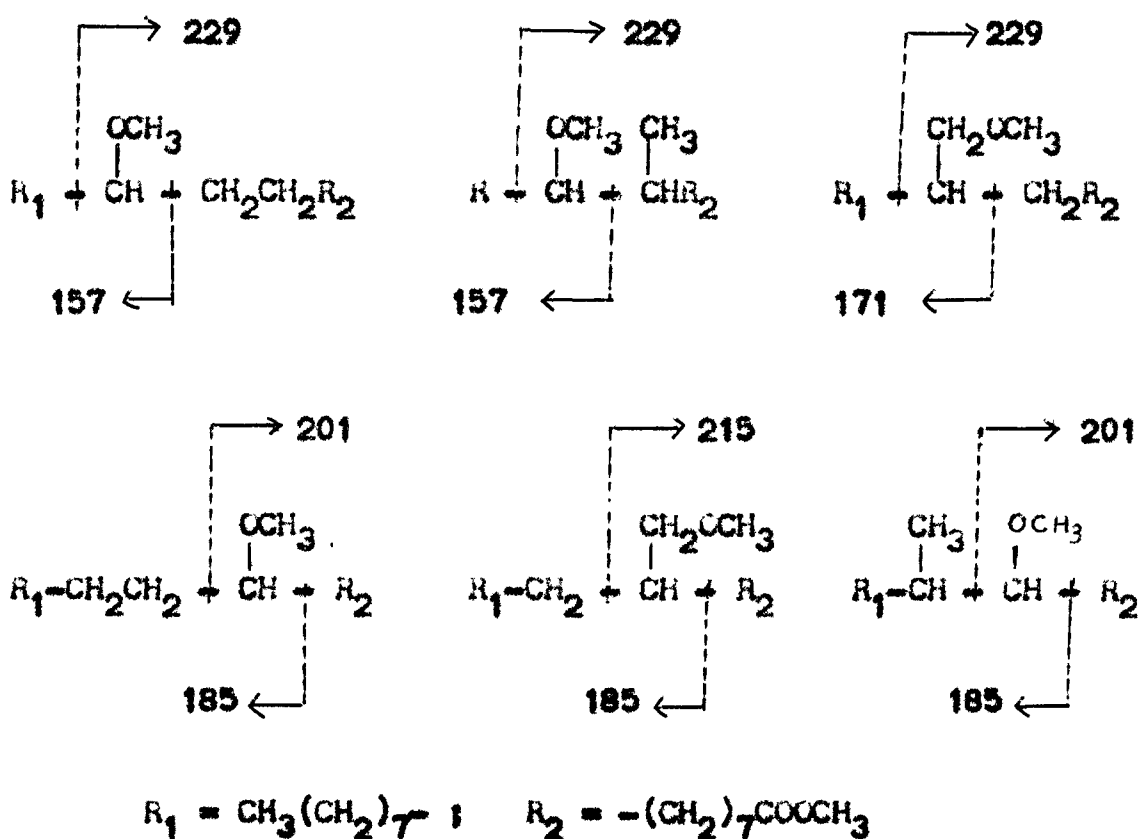
acids⁴⁷. Gensler and coworkers^{39,41} applied the chromium trioxide oxidation technique to a synthetic dicyclopropyl ester (16), OCH₃ replacing N(CH₂)₄ and an analogous ester prepared by degradation of the mycolate from another strain of M. tuberculosis.



The mass spectra of the monoketones prepared from these esters contained diagnostic peaks, and those in the derivative of the natural compound led to the homologous structures for the parent mycolic acids in general agreement with those (19) found previously.

The structures (19) of the mycolic acids from M. tuberculosis, determined by the chromium trioxide oxidation procedure^{41,47} supported similar structures found previously⁴⁸ by application of the BF₃-MeOH ring cleavage procedure (Scheme 2). Simple cyclopropane esters such as methyl cis-9,10-methyleneoctadecanoate reacted with 50% BF₃ in methanol to yield all the possible six isomeric methoxy esters (20) and a complex mixture of unsaturated esters¹¹. The mass spectra of

the isomeric methoxy esters (20) contained six intense peaks corresponding to the expected cleavages adjacent to the oxygenated functions.



(20)

A dicyclopropyl mycolate from M. tuberculosis was pyrolysed to give a dicyclopropyl merxaldehyde, which was then reduced to an alcohol. The methanesulphonate was prepared and this derivative reduced with lithium aluminium deuteride to yield the 1-deuterio dicyclopropyl alkane⁴⁸. Reaction of this

hydrocarbon with BF_3 in methanol gave methoxy and dimethoxy derivatives whose mass spectra contained the expected diagnostic fragments leading to structures incorporated in formula (19). It should be noted that ⁵there structures^{41,47,48} for the mycolic acid of M. tuberculosis (19) differed significantly from those obtained earlier⁴⁹ by direct interpretation of the mass spectra of mycolic esters, showing that mass spectral cleavages adjacent to underivatized cyclopropane rings are unreliable. Cyclopropane rings in methoxy and keto mycolic acids from M. tuberculosis have also been located by application of the boron trifluoride-MeOH procedure to suitable derivatives, followed by mass spectrometry⁵⁰.

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